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Elevated Levels of Antibodies to Polyuridylic Acid Detected and Quantitated in Systemic Scleroderma Patients by Solid Phase Radioimmunoassay

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A solid support radioimmunoassay has been developed to detect immunoglobulin specific circulating antibodies to polyuridylic acid (Poly U), single-stranded RNA (ss RNA), and single-stranded DNA (ss DNA) in scleroderma and other connective tissue diseases. The assay system uses flex-vinyl microtiter plates on which bovine methyl albumin, the respective polynucleotide, a 1:80 dilution of patient serum, and tritiated high affinity anti-IgG, -IgA, or -IgM are layered. The individual wells containing the sandwich assay are then counted for the presence of labeled immunoglobulins and the results are reported in μg/ml.

Of the 30 scleroderma patients tested, only patients with diffuse systemic scleroderma had antibody levels reactive to Poly U >4.0 μg/ml and to ss RNA < 3.0 μg/ml. Patients with linear scleroderma or morphea had antibody levels to Poly U <3.0 μg/ml and very little antibody to ss DNA or ss RNA in their sera. Partial cross reactivity to Poly U was found only in SLE patients with high levels of Ab to ss DNA. Insignificant levels of Poly U antibody were found in patients with other connective tissue diseases and in normal controls.

High levels of serum antibody in patients which reacted with Poly U suggest active diffuse systemic scleroderma.

The etiology of scleroderma has been investigated from a variety of approaches including detection of abnormalities of the microvascular system [1–4] biochemical abnormalities of the collagen [5–8] and morphological changes [9–11]. Recently, a number of immunological abnormalities have been reported, such as the association of systemic scleroderma with autoimmune hemolytic anemia, graft vs. host disease, and the high incidence of antinuclear antibodies [12–15]. Antibodies to various types of RNA or RNA-protein have also been found in patients with scleroderma, systemic lupus erythematosus (SLE), Sjogren's syndrome, mixed connective tissue disease, and Raynaud's phenomenon [16].

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Abbreviations:
ANA: antinuclear antibody
AS: acrosclerosis
BAC: bromoacetyl cellulose
DS: diffuse scleroderma
FDNB: ^{3}H fluorodinitrobenzene
MBSA: methylated bovine serum albumin
RAST: radioallergosorbent
SLE: systemic lupus erythematosus
SPRIA: solid phase radioimmunoassay

Alarcon-Segovia and Fishbein [17] have shown that many scleroderma patients have antibodies that react with uracil-specific ssRNA as detected by counter-immunoelectrophoresis. Because uracil-specific ssRNA might be an immunological marker of the disease or involved in the disease process, we decided to investigate the incidence of antibodies to polyuridylic acid in the various connective tissue diseases. Using a solid state radioimmunoassay technique, we have detected a population of auto-antibodies which binds to the synthetic polynucleotide, polyuridylic acid (Poly U), in the sera of most patients with diffuse scleroderma (DS).

MATERIALS AND METHODS

Clinical Material

Patients having scleroderma were classified either as localized skin disease which included morphea and linear scleroderma, or systemic scleroderma. The patients having systemic scleroderma were further subdivided into (1) diffuse scleroderma (with skin involvement of more than face and distal extremities) or (2) acrosclerosis (AS) with Raynaud's phenomenon and skin involvement restricted to the face and/or distal extremities. The disease of these patients was classified as active when new organ systems became involved, skin induration (hide binding) increased, and/or involved surface area increased.

Patients were classified as having Raynaud's disease only when there were absolutely no clinical signs or symptoms of possible underlying connective tissue disease. Patients were classified as having acrosclerosis if there was no more cutaneous involvement than the distal extremities and/or the face. Patients with other connective tissue diseases were included in this paper only if their diagnosis had been well established by appropriate clinical and laboratory criteria. The actual numbers of patients with the various connective tissue disease tested are contained in the results section.

Venous blood was drawn from patients with various connective tissue diseases and from normal controls. The samples of patients' sera, taken at various times during the course of the disease, were received within 6 hr after drawing, and the sera was separated by centrifugation at 4°C. Samples were stored at −5° to −10°C and thawed once before testing. Sera were selected for testing when the patient's disease appeared to be most immunologically active.

Radioallergosorbent (RAST) Buffer 10X Solution

Thirty grams of Dextran T 70 (Pharmacia) were dissolved in 500 ml of 0.9% saline. The remaining reagents were then dissolved in their respective order: 85 gm $NaH_2PO_4 \cdot H_2O$; 85.5 gm NaCl; 3.0 gm NaN_3; 50 ml Tween 20; volume was brought up to 1000 ml with distilled H_2O and to a pH of 7.2. The working solution used was a 1:10 dilution (distilled H_2O) of the above with a pH of 7.2.

Single-stranded Polyuridylic Acid (Poly U, RNA and DNA)

Single-stranded Poly U was made from double-stranded Poly U (Sigma) by dissolving (1 mg/ml) in distilled water and bringing up to volume (1 mg/10 ml) with RAST buffer pH 7.2. The solution was heated at 100°C for 12 min, then quickly cooled in ice water. This same method was used to obtain the single-stranded forms of RNA (Type II Sigma) and DNA (Type I Sigma). Partial reannealment of less than 5% may occur in these nucleic acids [18].

High Affinity Anti-IgG

Pooled normal serum was fractionated with a 15% final volume Na_2SO_4 solution to precipitate the immunoglobulins, which were then

dissolved to original volume with distilled H_2O and chromatographed on a G-200 Sephadex column to obtain the IgG fraction. The IgG fraction was bound to bromoacetyl cellulose (BAC) (approx. 45.0 mg IgG/gram of BAC) according to the method of Robbins [19]. After appropriate washing, goat anti-human IgG (Fc specific-Cappel) was incubated with the matrix (9.0 mg Ab/gm matrix) for 4 hr at 25°C with gentle shaking. After washing twice with 0.9% saline, the matrix was treated with pH 4.5 0.01 M acetate-buffered saline for 10 min, washed once with 0.9% saline, and was then treated with 0.017 M HCl-buffered saline pH 2.5 to remove high affinity anti-IgG. The eluted high affinity anti-IgG was then neutralized by adding 0.15 M $NaHCO_3$ and the concentration was determined by a modified Lowry protein determination [20] and double-immunodiffusion [21].

Both high affinity anti-IgM and -IgA were isolated by similar methods as that for high affinity anti-IgG, using goat anti-human IgM or IgA heavy chain specific anti-sera (Cappel). Human IgM was obtained by G-200 chromatography of at least 6 pooled patient sera samples after Na_2SO_4 precipitation. The IgA used as the antigen on immunoabsorbent matrix was obtained from a pool of the sera of 6 IgA myeloma patients.

^3H Labeling of High Affinity Anti-IgG

The general method of Sanger [22] was used to label high affinity anti-IgG with ^3H Fluorodinitrobenzene (FDNB). A solution of 500 μg anti-IgG/1.0 ml saline (0.9%) was raised to a pH of 9.0 by the addition of 2 ml of 0.15 M sodium borate-buffered saline. This solution is immediately added to benzene-evaporated FDNB and allowed to react for 2 hr on a rocker platform. Generally, 10 molecules of FDNB will label 1 molecule of anti-IgG sufficiently. Excess ^3H FDNB was removed by desalting with a PD-10 column (Pharmacia) and PBS pH 7.2 and total CPM were calculated per ng of anti-IgG.

Quantitation of Working Curve for RIA

Quantitation of working curves of patients' anti-Poly U antibodies and ^3H labeled anti-immunoglobulins were obtained by making dilutions of both components in a grid manner. To find the best working dilution of the serum we used series of 2-fold dilutions from anti-Poly U positive (determined by double-diffusion and counter-immunoelectrophoresis) patients' sera ranging from 1:20 to 1:640 with a series of labeled anti-IgG concentrations ranging from 20 ng to 200 ng/well. Each series was plotted and the best working curve was selected. A 1:80 dilution of patients' sera provided a concentration of anti-Poly U antibodies that would not saturate all the antigenic sites of the Poly U. Similar titration curves were used to determine the IgM and IgA anti-Poly U antibody concentration in patients' sera.

Solid Phase Radioimmunoassay (RIA)

The solid phase microtiter radioimmunoassay of Tan and Epstein [23] was modified by incorporating the RAST technique for immunoglobulin specificity. Flexible, polyvinyl, 96-well microtiter U plates were etched 5 min per well with spectral-grade toluene, washed 3 times with PBS pH 7.2, and once with distilled H_2O. The plate was coated with 0.1 ml methylated bovine serum albumin (MBSA) per well (1 mg/10 ml H_2O), incubated for 4 hr at room temperature, and washed 3 times with PBS pH 7.2. Single-stranded Poly U, single-stranded RNA, or single-stranded DNA (10 μg/0.1 ml RAST) was applied to the MBSA coated wells and incubated at 4°C for 12 hr followed by 6 washings with RAST buffer.

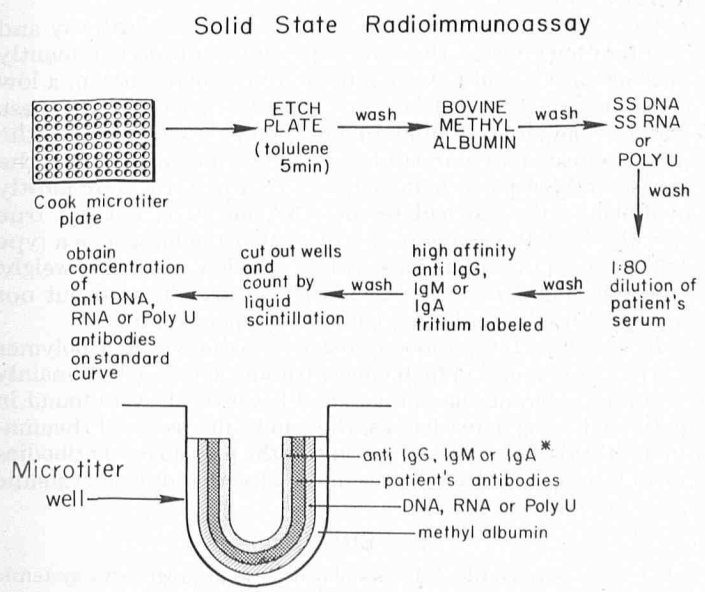

FIG 1. The sandwich assay system of the radioimmunoassay.

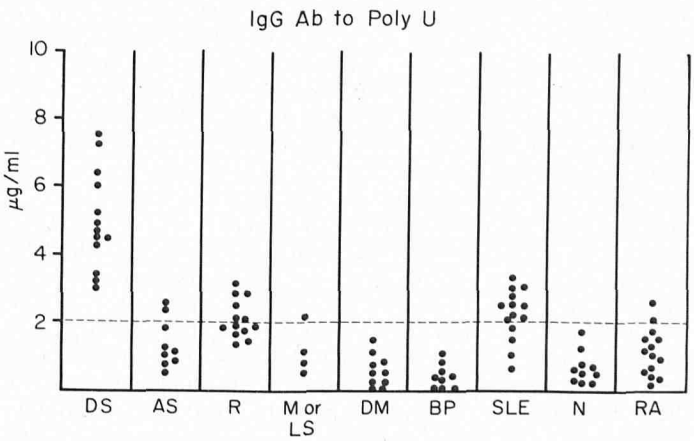

FIG 2. Comparisons of concentrations of IgG antibodies to Poly U in the various connective tissue diseases. *DS*=diffuse scleroderma; *AS*=acrosclerosis; *R*=Raynaud's disease; *M*=morphea; *LS*=linear scleroderma; *DM*=dermatomyositis; *BP*=bullous pemphigoid; *SLE*=systemic lupus erythematosus (active); *N*=normal human serum; *RA*=rheumatoid arthritis. The antibody levels to Poly U of active diffuse scleroderma were significantly higher ($p > .001$) than levels of the other types of scleroderma or of other connective tissue diseases, with Raynaud's disease being significantly different ($p > .01$) from the normals.

Patients' sera (0.1 ml/well) of a 1:80 dilution in RAST buffer were added to the respective wells in duplicate and incubated at room temperature for 4 hr followed by 6 washings with RAST buffer. High affinity labeled anti-IgG, -IgM or -IgA (0.1 ml/well: 40 ng/ml unlabeled and 40 ng/ml labeled Ab) was added to the respective wells, incubated for 12 hours at room temperature and washed 6 times with RAST buffer. The wells were cut from the plate and placed in scintillation vials. After liquid scintillation cocktail (any type suitable for counting aqueous solutions and organic soluble samples) was added to the vials, they were allowed to stand one hour at room temperature before counting for the presence of labeled anti-immunoglobulin. The counts per minute per ng were determined by counting a known volume of a dilution of the labeled anti-IgG, -IgM or -IgA. With the CPM/ng of type-specific anti-immunoglobulin established the serum sample was calculated as micrograms of IgG, IgM and/or IgA to ss Poly U, ss DNA or ss RNA/well by a linear regression plot (Fig 1).

RESULTS

Ten of the 13 patients with diffuse scleroderma had IgG antibody reactive with Poly U greater than 4.0 μg/ml as illustrated in Fig 2. Eight of these 10 patients were actively developing new skin lesions or had increased plaque induration at the time the serum samples were obtained. The 2 other patients of this group were found to have been dermatologically more active 2 years prior to obtaining the sera. Eight of these 10 patients had Raynaud's phenomenon and sclerodactyly and 5 of the 10 had multisystem involvement of heart, lung and/or kidney. Only 1 of the 3 diffuse scleroderma patients that had anti-Poly U antibody levels of less than 4 μg/ml had active disease; in fact, this patient was very active with rapidly developing renal disease.

No highly significant levels of IgG antibodies reactive with Poly U (>4.0 μg/ml) were found in patients in any of the other disease studied including the patients with acrosclerosis, linear scleroderma, and morphea. Smaller amounts of IgG reactive with Poly U (<4.0 μg/ml and >2.0 μg/ml) were found in some patients with SLE, rheumatoid arthritis, acrosclerosis, Raynaud's disease, and 1 patient with linear scleroderma. This patient with linear scleroderma subsequently developed a sig-

nificant anti-Poly U level. We found no detectable serum IgA antibodies to Poly U in any of the patients tested.

Three of 12 patients with diffuse scleroderma showed IgG antibody to single-stranded RNA greater than 4.0 μg/ml (Fig 3). Four of 18 patients with SLE also showed IgG antibody to single-stranded RNA greater than 4.0 μg/ml. These IgG antibodies could possibly be a result of either cross reactivity at anti-Poly U antibodies with RNA or the presence of antibodies in patient sera to many different nucleic acids. The actual specificity of all the individual antibodies was not studied.

Only patients with active systemic lupus erythematosus with renal involvement (13 of 13) showed IgG antibody to single-stranded DNA greater than 4.0 μg/ml (Fig 4). Sera of patients with all other connective tissue diseases showed IgG Ab to single-stranded DNA considerably below 4.0 μg/ml, indicating little cross-reactivity between antibodies to Poly U found in DS patients and DNA.

The patients with diffuse scleroderma had antinuclear antibody (ANA) patterns mainly consisting of the particulate patterns but not true speckles [15]. Only 1 patient had a serum ANA pattern of nucleolar, and 2 patients were negative.

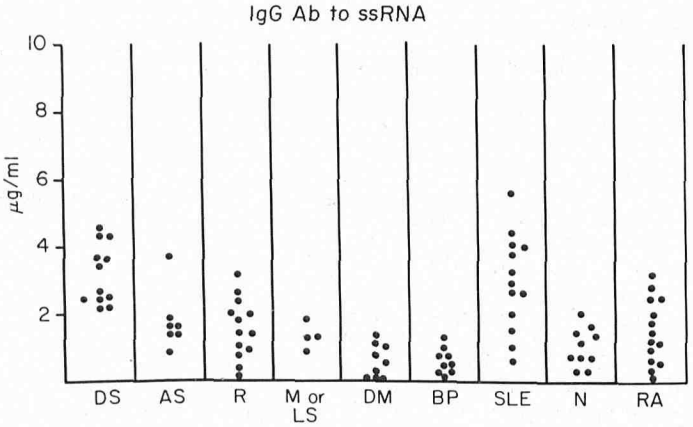

FIG 3. Comparison of IgG antibody concentration to ss RNA in the various connective tissue diseases. No real differences between diseases were noted with the exception of active diffuse scleroderma ($p > .001$). *DS*=diffuse scleroderma; *AS*=acrosclerosis; *R*=Raynaud's disease; *M*=morphea; *LS*=linear scleroderma; *DM*=dermatomyositis; *BP*=bullous pemphigoid; *SLE*=systemic lupus erythematosus (active); *N*=normal human serum; *RA*=rheumatoid arthritis.

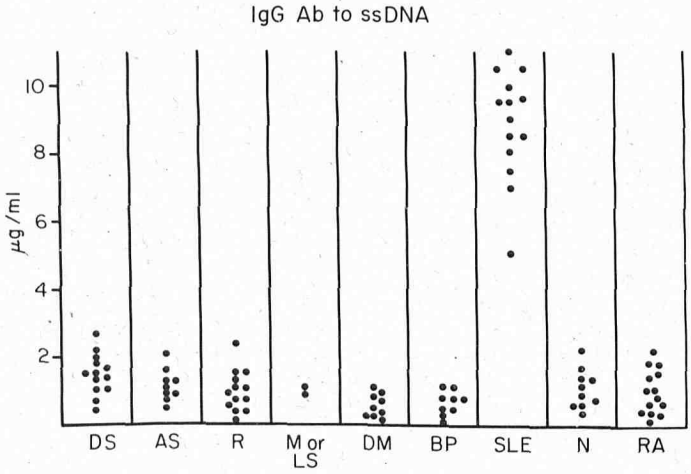

FIG 4. Comparison of IgG antibody concentrations to ss DNA in the various connective tissue diseases. The antibody concentration to ss DNA of the active SLE patients was significantly different ($p > .001$) from that of the other connective tissue diseases. *DS*=diffuse scleroderma; *AS*=acrosclerosis; *R*=Raynaud's disease; *M*=morphea; *LS*=linear scleroderma; *DM*=dermatomyositis; *BP*=bullous pemphigoid; *SLE*=systemic lupus erythematosus (active); *N*=normal human serum; *RA*=rheumatoid arthritis.

DISCUSSION

The use of a solid support system to fix the antigen Poly U provides a sandwich assay to detect and quantitate the type of reactive antibody. Since methylated bovine serum albumin enables ss DNA, ss RNA or ss Poly U to be fixed to a solid matrix, it eliminates the need for precipitating a complex found in other assays and prevents the reannealment of single-stranded nucleic acid to the double-stranded form as has been previously established using tritiated nucleic acids [18]. The use of ^{3}H anti-IgG, -IgM or -IgA provides the assay with immunoglobulin type specificity.

Our data illustrate that the majority of patients with active diffuse scleroderma have IgG antibodies which bind the synthetic polynucleotide, polyuridylic acid. These antibodies were not detected in any significant concentration in other types of scleroderma or connective tissue diseases, although small amounts were found in SLE and RA, which were possibly due to cross reactivity of heterogeneous anti-DNA or anti-RNA antibodies. Raynaud's disease patients with somewhat elevated levels of anti-Poly U antibody may be progressing to an active form of diffuse scleroderma or may merely have anti-RNA antibodies. We are continuing to monitor these patients to ascertain whether the Raynaud's disease may develop into diffuse scleroderma.

One phenomenon that is hard to explain is the lack of presence of IgM antibodies in those patients that would react with Poly U. When we evaluated active SLE patients who had antibodies to DNA, small amounts of IgM antibodies were present with the IgG antibodies. The lack of IgM antibodies to Poly U might be due to the fact that high affinity IgG antibodies are necessary to react with this antigenic site in the test. Low affinity antibodies which are usually IgM may be lost in the vigorous washing procedure.

Alarion-Segovia and Fishbein [17] used counter-immunoelectrophoresis to detect the reactivity of various serum antibodies in patients with systemic scleroderma and SLE. They found that the majority of antibodies to RNA in scleroderma patients were reactive to uracil bases and not to other components of the RNA structure. All 40 of the scleroderma patients they studied gave precipitin lines to ss RNA and Poly U. In the solid phase assay, we use methylated bovine serum albumin as a binding agent between the vinyl well of the microtiter plate and ss Poly U. This binding is mainly by ionic charge and involves the ribose-phosphate components of Poly U, leaving the uridine bases open, thus allowing maximal antibody exposure. By using such a binding agent we believe the solid phase assay becomes very quantitative and qualitative as to which type of antibody reacts with Poly U.

The origin of the antigen is unclear. Pinnas, Northway and Tan [24] have tested the sera of patients with predominantly nucleolar ANA and have identified a nucleolar antigen, a low molecular weight 4-6s RNA, to which they react. Most of these patients had either scleroderma or Sjogren's syndrome. Of the patients tested in our study, only one had manifested the nucleolar ANA pattern; in fact, the ANA patterns were mostly confined to the particulates or ANA negative, but not true speckles [25]. We can only speculate that the antigen is a type of RNA high in uridine base. A few such low molecular weight RNAs localized to the nucleus, rich in uridylic acid but not produced by the nucleolus, have been reported [26].

In summary, IgG antibodies reactive to the synthetic polymer Poly U were found in high concentrations (>4.0 μg/ml) mainly in diffuse scleroderma patients and lower levels were found in patients having acrosclerosis, Raynaud's disease, and rheumatoid arthritis. Quantitative testing for the presence of antibodies to Poly U may aid in the diagnosis of active diffuse systemic scleroderma.

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